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Visualization of neurotransmitter uptake and release in serotonergic neurons

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HIGHLIGHTS

- We have established the visualization of fluorescent transmitter-like substrates in stem cell-derived serotonergic neurons.
- We have combined uptake and release of fluorescent substrates into and from serotonergic neurons with quantitative image analysis.
- These methods allow optical discrimination of axonal from somatodendritic transmitter release.

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ABSTRACT

Background: To study serotonergic volume neurotransmission at cellular level it needs to investigate neurotransmitter release and re-uptake sites in serotonergic neurons. However, due to the low number of cell bodies in the raphe nuclei and their widely branching neurites, serotonergic neuronal cultures are not accessible *ex vivo*.
New method: We have combined differentiation protocols for the generation of stem cell-derived serotonergic neurons together with confocal microscopy to study the uptake and release of fluorescent substrates known to be selectively taken up by monoaminergic neurons. These substances include: (i) 4-(4-(dimethylamino)styryl)-*N*-methylpyridiunium (ASP+), an analog of the neurotoxin MPP+; (ii) the fluorescent false neurotransmitter (FFN511); and (iii) serotonin (5-hydroxytryptamine; 5-HT) itself, which is known to emit fluorescence upon excitation at 320–460 nm.
Result: ASP+ is taken up into living serotonergic neurons through the serotonin transporter, but not accumulated into synaptic vesicles; FFN511 diffuses in a SERT-independent way into serotonergic neurons and accumulated into synaptic vesicles. KCl-induced release of FFN511 and 5-HT can be visualized and quantified in living serotonergic neurons.
Comparison with existing methods: Application of ASP+ so far has been used to investigate substrate/transporter interactions; studies on FFN511 uptake and release have only been performed in dopaminergic neurons; quantitative studies on uptake and release of 5-HT in living serotonergic neurons have not been reported yet.
Conclusion: The differentiation protocols for the generation of stem cell-derived serotonergic neurons combined with the application of different fluorescent dyes allow to quantify neurotransmitter uptake and release in living serotonergic neurons *in vitro*.

1. Introduction

Serotonin (5-hydroxytryptamine, 5-HT) is a widespread neurotransmitter in the central nervous system (CNS). Serotonergic neurons are located in raphé nuclei in the brain stem from where they project numerous axonal fibers into many brain regions such as cortical areas, the hippocampus, and the basal ganglia (Pineyro and Blier, 1999; Sur et al., 1996). The serotonergic system is involved in the modulation of mood, emotion, sleep, and appetite and contributes to learning and memory formation (Meneses and Liy-Salmeron, 2012).

In the recent years mounting evidence has been provided that serotonergic neurons modulate synaptic plasticity more importantly *via* extrasynaptic axonal and somatodendritic neurotransmitter release rather than through synaptic signaling (Adell

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et al., 2002; De-Miguel and Trueta, 2005; Hornung, 2003; Vizi et al., 2010). This "volume transmission" is regulated by the activity of the serotonin transporter (SERT), which defines the extracellular concentration of serotonin (Lau and Schloss, 2012), and of 5-HT autoreceptors which control the neuronal firing rate (Vizi et al., 2010). This implies that high firing rates in the somatodendritic region can negatively control axonal transmitter release *via* activation of somatodendritic autoreceptors whereas high somatodendritic SERT activity diminishes somatodendritic autoreceptor activity and thereby elevates axonal 5-HT release (Kaushalya et al., 2008).

To get insight into the control of non-synaptic serotonergic neurotransmission it needs to study structural and functional aspects such as the anatomical distribution of both, axonal and somatodendritic uptake and release sites in serotonergic neurons. For many years such studies *in vitro* have been hampered by the lack of selective fluorescent substrates for living serotonergic neurons and because, until recently, serotonergic neuronal cultures were not accessible *ex vivo*.

With respect to substrates for monoaminergic neurons fluorescent compounds such as 4-(4-(dimethylamino)styryl)-*N*-methylpyridiunium (ASP+) have been developed (Mason et al., 2005). ASP+, a fluorescent analog of the neurotoxin MPP+, has been shown to be a substrate for the plasma membrane monoamine transporters for dopamine (DAT), norepinephrine (NET) and serotonin (SERT). Consequently ASP+ has been used to intensively analyze binding and transport activity of the NET, SERT and DAT in heterologous expression systems such as HEK293 cells or organotypic slice culture (Bolan et al., 2007; Inyushin et al., 2013; Mason et al., 2005; Oz et al., 2010; Schwartz et al., 2003). Very recently a new fluorescent monoamine analog (Fluorescent False Neurotransmitter, FFN511) has been synthesized which allowed to visualize dopamine release from individual presynaptic terminals in the striatum (Gubernator et al., 2009). In order to study neurotransmitter uptake and release into and from living serotonergic neurons *in vitro* we have adopted a method to derive serotonergic neurons from neuronal progenitor cells (Buc-Caron et al., 1990; Mouillet-Richard et al., 2000) as well as from mouse embryonic stem cells (Lau et al., 2010). In the present study we have quantified the uptake and release of ASP+, FFN511 and 5-HT into living serotonergic neurons *in vitro*. For the latter, we have exploited the fact that at higher concentrations the natural substrate 5-HT is excited at wavelengths from 320 to 460 nm and can be detected at wavelengths from 390 to 540 nm (Crespi et al., 2004).

2. Materials and methods

2.1. Maintenance and serotonergic differentiation of 1C11 cells and mouse embryonic stem (ES) cells

1C11 cells were obtained from Dr. Odile Kellermann (Paris, France) and maintained in Dulbecco's modified Eagle's medium supplemented with 10% FCS, penicillin (100 U/ml), and streptomycin (100 μg/ml). The serotonergic differentiation of 1C11 cells (1C11$^{5\text{-HT}}$-cells) was induced by addition of 1 mM dibutyryl cAMP and 0.05% cyclohexane carboxylic acid (Mouillet-Richard et al., 2000). For each differentiation, 5000 cells per well were plated in μ-slide 8 wells or μ-slide I Luer chambers for flow applications (Ibidi). The serotonergic differentiation was completed after 4 days and resulted in 100% 1C11$^{5\text{-HT}}$-neurons, which were positive for serotonergic markers such as SERT, vMAT2 and tryptophan hydroxylase 2 (TPH2) but did not express the monoamine transporters DAT and NET.

For a detailed description for generating mouse ES cell-derived serotonergic neurons refer to (Lau et al., 2010). Briefly, serotonergic ES cell differentiation comprises three stages: (i) generation of neurostem spheres, (ii) proliferation and selection of neuronal precursors, and (iii) terminal differentiation. Functional and terminally differentiated ES cell-derived serotonergic neurons (ES$^{5\text{-HT}}$-cells) can be obtained in 14 days. About 90% of the resulting neurons exhibited a serotonergic phenotype as judged by immunostaining against serotonin, TPH2, SERT as well as 5-HT1A and 5-HT1B autoreceptors. Immunostaining for DAT and NET was always negative on all neurons obtained with our differentiation protocol ruling out the possibility of the development of other monaminergic neurons or co-expression of DAT and NET on ES$^{5\text{-HT}}$-neurons.

2.2. Confocal laser scanning microscopy

All images were acquired using a Leica TCS SP5 microscope using an HCX PL APO 63.0× oil planchromat lens with an NA 1.40. Images were acquired as *z*-stacks (0.5 μm sections), single plane (*xy*) time series or *z*-stack (*xyz*) time series were recorded using an argon ion laser at 458 nm wavelength (FFN511, Abcam Chemicals; 5-HT, Sigma) or a DPSS laser at 561 nm wavelength (ASP+, Sigma). For image acquisition, 5-HT was excited at the far end of its excitation spectrum and detected in the 460–540 nm range (Crespi et al., 2004). Serotonergic neurons were incubated in medium without serum with different concentrations of ASP+ (5, 10, 30, 50 μM), FFN511 (1 μM, 2.5 μM, 5 μM and 10 μM, *xy*/*xyz t*-series were acquired for 3 min), or 5-HT (100 μM, 250 μM, 500 μM; *xy*/*xyz t*-series were acquired for 5 min). Vesicular monoamine transporter vMAT2 blocker RO4-1284 (Sigma–Aldrich) was applied at 10 μM for 15 min (only *z*-stacks acquired). For live cell imaging, serotonergic neurons were incubated with 10 μM FFN511 for 3 min. All images were acquired using a heated stage (Ibidi Heating System 2, Ibidi) and media were exchanged using a KDS 100 syringe pump (KD Scientific).

2.3. Blocking experiments

2.3.1. Blocking of SERT with escitalopram

Serotonergic neurons were incubated in medium with 20 μM escitalopram (Lundbeck) for 2 h at 37 °C (Lau et al., 2009). Afterwards they were loaded with either ASP+ (30 μM), FFN511 (5 or 10 μM) or 5-HT (500 μM) in the presence of 20 μM escitalopram. Subsequently confocal *z*-stacks of the cells were recorded. Prior to image acquisition, all cells were washed three times with dye-free medium to reduce background staining.

2.3.2. Blocking of the vesicular monoamine transporter vMAT2 with the Ro 4-1284

Serotonergic neurons were incubated in medium with 30 μM Ro 4-1284 for 30 min at 37 °C. Afterwards they were loaded with either ASP+ (30 μM), FFN511 (10 μM) or 5-HT (500 μM) in the presence of 30 μM Ro 4-1284 for 15 min. Confocal *z*-stacks were acquired. Prior image acquisition the cells loaded with FFN511 were washed three times with medium devoid of FFN.

2.4. Exocytosis experiments with KCl

Serotonergic neurons were loaded with either ASP+ (30 μM), FFN511 (10 μM) or 5-HT (100 μM) for 3–15 min. Prior to KCl-induced depolarization FFN511-loaded cells were washed three times with medium. Depolarization was induced by application of medium supplemented with 40 mM KCl and 2 mM $CaCl_2$. 5-HT-loaded cells were imaged in the presence of 20 μM escitalopram. Confocal *z*-stacks were acquired before and after application of high potassium media.